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An Increase in Acid Resistance of Foot-and-Mouth Disease Virus Capsid Is Mediated by a Tyrosine Replacement of the VP2 Histidine Previously Associated with VP0 Cleavage

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The foot-and-mouth disease virus (FMDV) capsid is highly acid labile, but introduction of amino acid replacements, including an N17D change in VP1, can increase its acid resistance. Using mutant VP1 N17D as a starting point, we isolated a virus with higher acid resistance carrying an additional replacement, VP2 H145Y, in a residue highly conserved among picornaviruses, which has been proposed to be responsible for VP0 cleavage. This mutant provides an example of the multifunctionality of picornavirus capsid residues.

The picornavirus foot-and-mouth disease virus (FMDV) is the etiological agent of a highly contagious disease that affects cloven-hoofed animals, including important livestock species (1, 2). FMDV virions are arranged in an icosahedral capsid built by 60 copies of each of the four structural proteins (VP1 to VP4) arranged into 12 pentameric subunits that constitute the intermediates for capsid assembly and disassembly (3–5). The FMDV capsid disassembles into pentameric subunits at mildly acidic pH. This property of the capsid is key for the viral RNA uncoating that takes advantage of endosomal acidification during virus internalization in host cells (6–9). We previously reported that a single amino acid substitution at the N terminus of VP1 protein (VP1 N17D) selected in mutant m6 after treatment with pH 6.0 conferred increased resistance to acidic pH to type C FMDV isolate C-S8c1 (10). Similar findings have been recently described for type O FMDV (11). Here we report the isolation and characterization of a novel mutant from m6 population that displays an even higher degree of resistance to acidic pH.

About 2×10^6 PFU of m6 mutant was incubated 30 min in phosphate-buffered saline (PBS) at pH 5.2, pH was neutralized with 1 M Tris at pH 7.5, and these samples were used to infect BHK-21 cells in semisolid agar medium (10). Individual plaques developed after 24 h of infection were amplified by infection in liquid medium (48 h). The viral populations recovered were subjected to treatment with pH 5.2 and amplified again (48 h). One of them, termed sr1, displayed a marked increase in its resistance to treatment with acidic pH compared to m6 and C-S8c1 (Fig. 1A), with pH_{50} values—defined as the pH that causes a loss of 50% of the infectivity (9)—of 5.4, 6.1, and 6.75 for sr1, m6, and C-S8c1,

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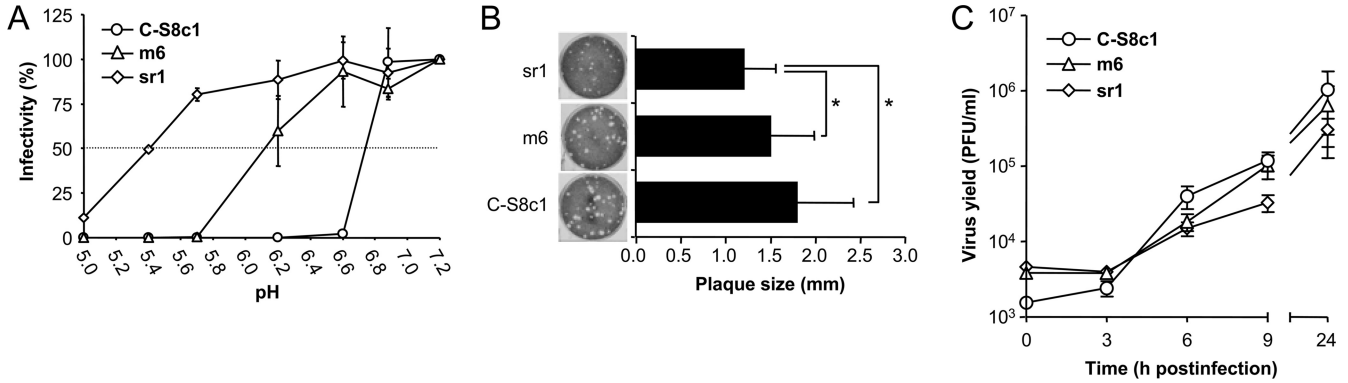

FIG 1 FMDV mutant sr1 displays an increased resistance to acidic pH without major effects on virus growth. (A) Acid sensitivity of C-S8c1, m6, and sr1 viruses. Equal amounts (PFU) of the different viruses were treated 30 min at room temperature with PBS at different pHs, as described previously (9). The samples were then neutralized with Tris at 1 M and pH 7.5 and added to BHK-21 monolayers. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.5. The intersections between the inactivation curves and dashed line indicate pH_{50} values; see the text for details. (B) Analysis of viral plaque size of C-S8c1, m6, and sr1. BHK-21 cells were infected in agar semisolid medium, and plaques were visualized by staining with crystal violet. About 100 viral plaques were analyzed for each virus. Asterisks (*) denote statistically significant differences (analysis of variance [ANOVA] $P < 0.05$). (C) Single-step growth curve analysis of C-S8c1, m6, and sr1. BHK-21 cells were infected (multiplicity of infection [MOI] of 1 PFU/cell), and the virus titer in the supernatants was determined by plaque assay at different times postinfection. Data are presented as means ± standard deviations.

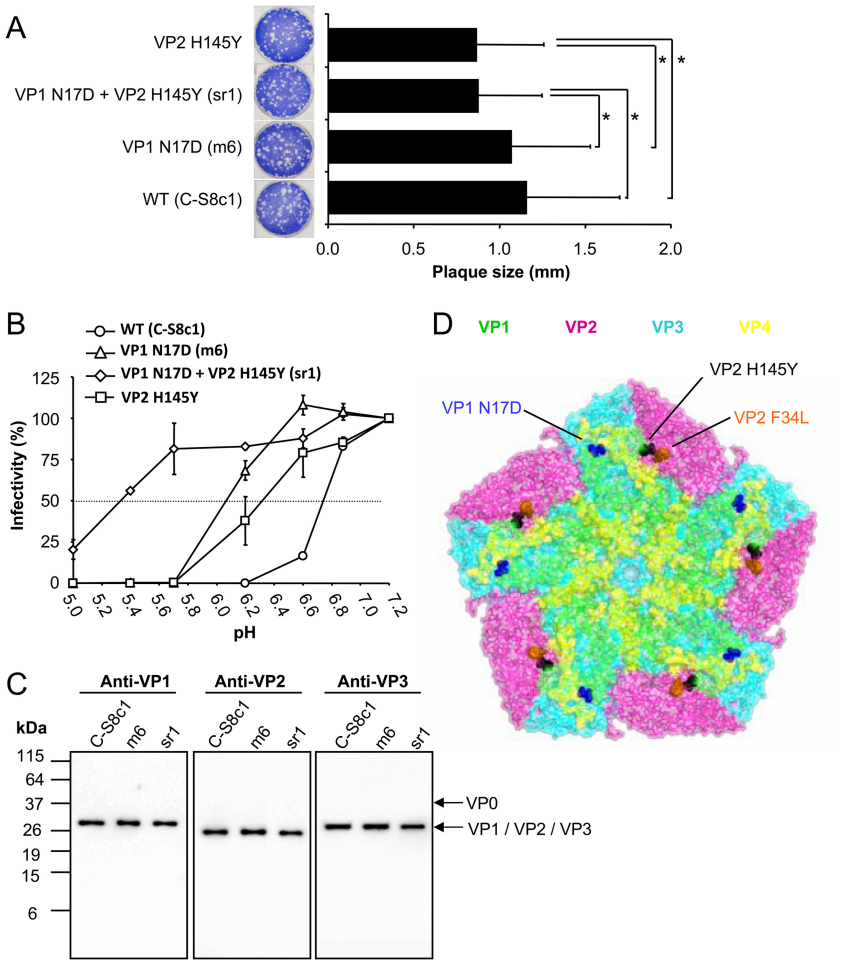

FIG 2 Amino acid substitution VP2 H145Y increases acid resistance of FMDV. (A) Analysis of plaque size of viruses recovered from infectious clone pMT28 (WT) and its derivatives encoding different amino acid substitutions found in mutant m6 (VP1 N17D) or mutant sr1 (VP1 N17D + VP2 H145Y) or VP2 H145Y alone. BHK-21 cells were infected in agar semisolid medium, and plaques were visualized by staining with crystal violet. About 100 viral plaques were analyzed for each virus. Asterisks (*) denote statistically significant differences (ANOVA $P < 0.05$). (B) Acid sensitivity of viruses recovered from infectious clone pMT28 and its derivatives shown in panel A. Equal PFU of the different viruses were treated and plated on BHK-21 monolayers as described for Fig. 1. Infectivity was calculated as the percentage of PFU recovered at each different pH relative to that obtained at pH 7.5. The intersections between the inactivation curves and dashed line indicate pH_{50} values; see the text for details. (C) Western blot analysis of purified virions of C-S8c1, m6, and sr1. Virions were purified from tissue culture supernatants through a 20% sucrose cushion followed by centrifugation in a 7.5% to 30% sucrose density gradient (9). The peak fraction containing the virions was loaded in 12% SDS-PAGE and analyzed by Western blotting using monoclonal antibody SD6 (anti-VP1), anti-VP2 (4A3), or anti-VP3 (6C2) (25, 26). The expected migration of VP0 (26) is indicated by an arrow. (D) Location on the structure of the C-S8c1 capsid (5) of amino acid residues found to be substituted in FMDV mutants with increased resistance to acid inactivation. An inside schematic view of a pentameric subunit in the capsid is shown. VP1 is green, VP2 is magenta, VP3 is cyan, and VP4 is yellow.

respectively. Thus, mutant sr1 displayed an increase of more than 1 pH unit in resistance to acidic pH compared to the parental C-S8c1. Mutant sr1 showed only a slight reduction in plaque size compared with those of m6 and CS8c1 (Fig. 1B), and no major differences in their viral growth curves were noticed (Fig. 1C).

Sequencing of the capsid coding region, as described in references 9 and 10, revealed that the sr1 population retained the nucleotide substitution A3256G leading to replacement VP1 N17D present in mutant m6 and had acquired substitution C2329T leading to replacement VP2 H145Y as the only changes relative to C-S8c1 (12). Infectious clones encoding the complete genomic sequence of C-S8c1 virus (13) were engineered to carry substitution VP2 H145Y either alone or combined with replacement VP1 N17D, and the corresponding viruses were recovered by transfection of BHK-21 cells with *in vitro*-synthesized viral transcripts (9, 10). Mutant VP2 H145Y and mutant VP1 N17D + VP2 H145Y showed a plaque size slightly smaller than those of VP1 N17D and the wild type (WT) (C-S8c1) (Fig. 2A), and no reversion to the parental sequence or further substitutions were detected in the capsid region of any of these viruses upon 6 passages. Relative to the C-S8c1 virus recovered from the infectious clone (pH_{50} of 6.75), VP2 H145Y replacement induced an increase in resistance to acidic pH slightly lower (pH_{50} of 6.3) than that of VP1 N17D (pH_{50} of 6.05, comparable to that of m6) (Fig. 2B). These increases were lower than that observed for the double mutant VP1 N17D + VP2 H145Y (pH_{50} of 5.35, comparable to that of sr1), indicating an additive effect of replacements VP1 N17D and VP2 H145Y on FMDV resistance to acidic pH. VP2 H145 (equivalent to poliovirus H195) is a highly conserved residue among picornaviruses that has been proposed to promote the autocatalytic RNA-dependent cleavage of the capsid precursor VP0 into VP2 and VP4 (14, 15). In poliovirus, substitution of H195 by threonine, argi-

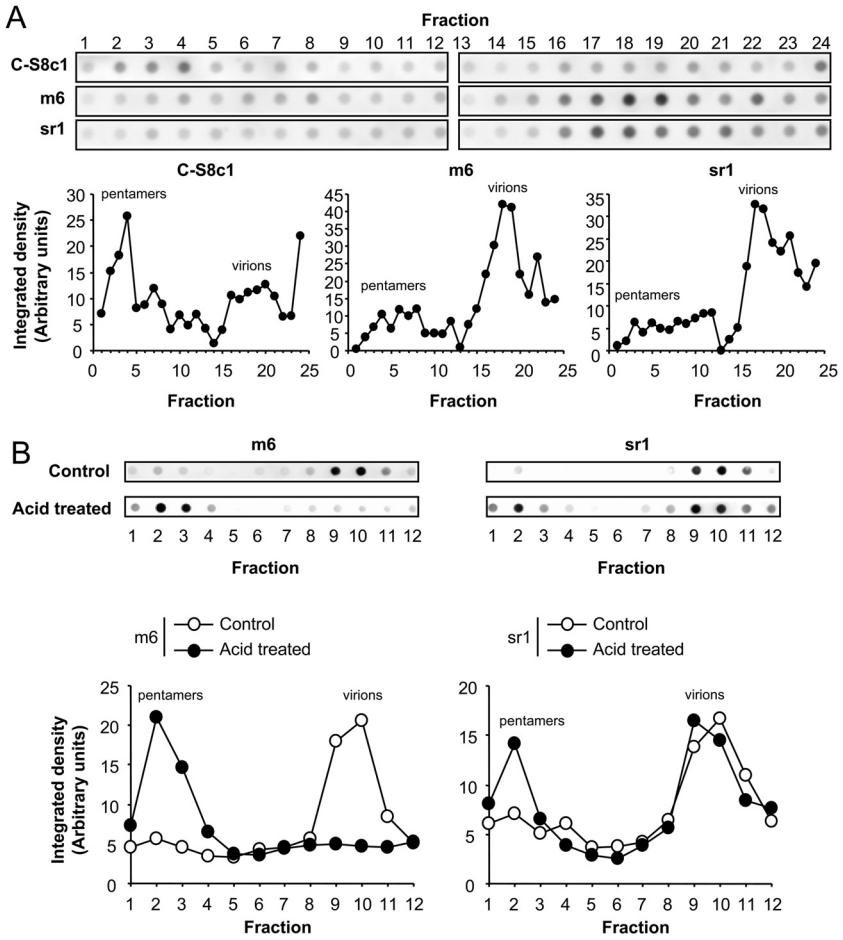

FIG 3 Mutant sr1 virions display increased stability at 4°C and acidic pH. (A) Analysis of the stability of virions of C-S8c1, m6, and sr1 at 4°C. Virions from C-S8c1, m6, and sr1 were purified from tissue culture supernatants as described for Fig. 2 and then dialyzed against PBS at pH 7.4. Purified virions were stored at 4°C for 15 weeks. The proportion of virions and pentamers (sedimentation coefficients of 140S and 12S, respectively) in each sample was determined by ultracentrifugation of the samples in a 7.5% to 45% sucrose density gradient (9) and analysis of fractions collected from the top of the gradients by dot blot using monoclonal antibody SD6 (anti-VP1) and chemiluminescence detection. Quantification of the integrated density in each dot corresponding to a fraction of the gradient is shown in the graphs. (B) Analysis of virion integrity after acid treatment. Purified virions of sr1 and m6 mutants prepared and stored as described for panel A were incubated with PBS at pH 7.2 (control) or 6.2 (acid treated) for 30 min at room temperature, and the proportion of virions and pentamers in each sample was determined by ultracentrifugation of the samples in a 7.5% to 45% sucrose density gradient and analysis by dot blotting using antibody SD6 and chemiluminescence detection. Quantification of the integrated density in each dot corresponding to a fraction of the gradient is shown in the graphs.

nine, glycine, or aspartic acid impaired VP0 cleavage and was lethal (14). Structural analyses suggest that the role of this histidine residue is conserved in both FMDV and poliovirus (15), albeit the cleavage of FMDV and that of poliovirus VP0 may differ in some aspects. Indeed, poliovirus empty capsids display VP0 (16), whereas FMDV empty capsids exhibit VP2 and VP4 (15). Our results show that no VP0 was detected in purified virions of mutant sr1 (Fig. 2C), suggesting that replacement of histidine by tyrosine does not impair its putative role in VP0 cleavage. VP2 H145 is located about 18 Å from VP1 N17D, close to an VP2 F34 residue (Fig. 2D) in which replacement F34L was found accompanying substitution VP1 N17D in another acid-resistant mutant (10). This indicates the importance of this region for acid resistance. VP2 H145 establishes a hydrogen bond with VP2 T33 and is close to residues 6 to 9 at the N terminus of VP1, a region of the capsid also involved in acid resistance (10, 17). The protonation at mildly acidic pH (about 6.5) of two histidines of VP3 located near the border of the pentameric subunit has been associated with electrostatic repulsions between neighbor pentamers that induce capsid disassembly (18, 19). The pKa of 5.25 for VP2 H145 side chain, calculated with PROPKA (20), is compatible with its protonation at more acidic pH, which should be abolished by replacement VP2 H145Y. Since H145 is located in an internal region of the capsid at the intraprotomeric interface, not at the pentameric interface, replacement VP2 H145Y could also impact the protomer conformation, affecting pentamers and hence capsid stability. Indeed, mutations in central regions of intermediates for capsid assembly can affect assembly of viral capsids (21).

The acid resistance of mutant m6 correlated with a lower sensitivity to heat inactivation relative to C-S8c1 virus (10). Thermal stability of FMDV virions is important for the immunogenicity of FMDV vaccines based on chemically inactivated virus whose efficacy is reduced when the cold chain (4°C) is broken or upon long-term storage (22, 23). Virions of C-S8c1, m6, and sr1 were purified by ultracentrifugation in sucrose gradients (9, 10) and stored at 4°C. After 15 weeks of storage, C-S8c1 virions had completely dissociated into pentameric subunits whereas m6 and sr1 preparations conserved a high proportion (about 80%) of virions

(Fig. 3A), confirming an increase in thermal stability at 4°C that could be advantageous for vaccine design. When the acidic sensitivity of these preparations was tested (Fig. 3B), an increased resistance to acid-induced dissociation of sr1 relative to m6 was noticed.

To analyze the effect of VP1 N17D and VP2 H145Y on virus antigenicity, we used an enzyme immunodot assay (24) that included a representative panel of virus-neutralizing monoclonal antibodies directed against different epitopes located at the antigenic sites identified in C-S8c1—site A (SD6), site C (7JA1), and site D (1G5, 2E5, and 5C4) (5). Relative to the parental virus, viruses carrying amino acid replacement VP1 N17D or VP2 H145Y or both replacements were recognized by all the antibodies in similar manners (data not shown). Further work, including the analysis of the immunogenicity of these mutants in natural hosts, is required to address the potential of these mutations to increase the stability of FMDV vaccines.

In summary, replacement VP2 H145Y confers resistance to acidic pH, leading to higher levels of resistance when combined with substitution VP1 N17D. Replacement H145Y did not result in major impairment of processing of either VP0, as no VP0 was detected in virions carrying this mutation, or virus growth. Our data provide unexpected evidence of the multifunctionality of this residue in the FMDV capsid.

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